FROM THE INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF UPPSALA, UPPSALA, SWEDEN

Ion Exchange Chromatography of Proteins and Some Applications to the Study of Different Phosphoesterases

by Hans G. Boman

AWE

FROM THE INSTITUTE OF BIOCHEMISTRY, UNIVERSITY OF UPPSALA.
UPPSALA, SWEDEN

Ion Exchange Chromatography of Proteins and Some Applications to the Study of Different Phosphoesterases

By HANS G. BOMAN
FIL. LIC., STOCKHOLMS NATION

INAUGURAL DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY, WHICH BY DUE PERMISSION OF THE FACULTY OF SCIENCE OF THE UNIVERSITY OF UPPSALA WILL BE PUBLICLY DISCUSSED IN THE LECTURE HALL OF THE INSTITUTE OF CHEMISTRY, MAY 23, 1958, AT 10 A.M. IN ADDITION TO THE PRESENT SURVEY THE PAPERS NUMBERED *8*, *9*, *10*, *11*, *12*, *13*, *15* AND *16* IN THE LIST OF REFERENCES WILL BE THE SUBJECT OF THE DISCUSSION

UPPSALA 1958
ALMQVIST & WIKSELLS BOKTRYCKERI AB

From the Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

Ion Exchange Chromatography of Proteins and Some Applications to the Study of Different Phosphoesterases

By Hans G. Boman

CONTENTS

Introduction 1
Ion Exchange Chromatography of Proteins 2
I. General 2
1. Experimental details 4
II. Starting-agent development 4
III. Gradient development 5
IV. Step-wise development 6
1. One step with negligible interaction between developer and adsorbent 7
2. One step with evident interaction between developer and adsorbent 9
3. Development with several steps 10
V. Chromatographic homogeneity 11
1. Artifacts 11
VI. Concluding remarks 12
Chromatographic Studies of Some Phosphoesterases 13
I. Acid phosphomonoesterases 13
II. Phosphodiesterases 15
Summary 16
References 16

INTRODUCTION

During the last five years, chromatography has found an increasing application in enzyme research. As the field is new, a general discussion of the methods used may serve some purpose. Recently two rather complete reviews were published by Moore and Stein (39) and by Sober and Peterson (60) and the first part of the present paper has therefore been limited to a discussion of the different ways in which a chromatogram of a protein mixture can be developed. The second part of the paper

is a summary of some applications to the study of different phosphoesterases, a field which sometimes has been regarded as one of the under-developed areas of enzyme chemistry.

It has earlier been found practical to divide chromatography into elution, frontal and displacement analysis (65). However, the terms "elution" and "displacement" have become associated with different mechanisms in chromatography. As the process behind protein chromatography appears rather complex and non-ideal, the present terms for the different procedures will be used here entirely as descriptions of the experimental methods by which the chromatograms were developed. On this basis, the procedures have been divided into the following three groups: (*a*) Starting-agent development, when the same solution is used for the equilibration of the column and for the development of the chromatogram; (*b*) gradient development, when the chromatogram is developed with the equilibrating agent to which a continuously increasing amount of a substance is supplied; (*c*) step-wise development, when different solutions are used for the equilibration of the column and the development of the chromatogram. It will be evident from the following that this is not a clear-cut classification, but it is felt that the results so far obtained with the different procedures are different enough to justify the present organization of the material.

ION EXCHANGE CHROMATOGRAPHY OF PROTEINS

I. General

Several attempts have been made to define the separation process called chromatography, and this question has recently been discussed by Cassidy in his monograph (18). A specific characteristic for all procedures called chromatography is, however, that they utilize the distribution between two phases, one being stationary and the other moving. In the case of proteins, the phase system can either be a liquid-liquid (I) or a liquid-solid one (II).

I. The liquid-liquid partition chromatography, or "partition chromatography" as it usually is called, was invented by Martin and Synge in 1941 and its first application to proteins came ten years later when Martin and Porter used a cellusolve-salt-water system for chromatography of pancreatic ribonuclease (37). Some further applications have later been published by Porter (49, 50, 51).

II. The liquid-solid chromatography includes the classical procedure, the history of which leads back at least to Tswett's experiments. Following the common classification (18), the liquid-solid chromatography may be divided into adsorption chromatography (*A*) and ion exchange chromatography (*B*), and both of them have been applied successfully to the separation of proteins.

A. Several column materials have been used for adsorption chromatography of proteins and can be found in the table of Sober and and Peterson's review (60). From the number of applications, it appears that calcium phosphate may be a very suitable adsorbent for proteins. Although batch-wise operation with gels of this material has been an almost classical tool for protein chemists, Agner in 1938 (1), was the first to use it for column work. Different calcium phosphates have for several years been systematically studied at this Institute, and references can be found in the last report by Tiselius, Hjertén and Levin (67).

R. UNIV. BIBLIOTHEEK LEIDEN

TABLE I.

Some ion exchangers suitable for protein chromatography[1].

Ion exchanger	Functional group	Skeleton	Examples of application	Additional references
Cation exchangers				
Dowex 50	$-SO_3^-$	Polystyrene	Acid phosphatase (*8, 15*)	44
SM and SE cellulose	$-SO_3^-$	Cellulose	Serum (48); enolase (36)	
Amberlite IRC 50	$-COO^-$	Hydrocarbon	Ribonuclease (26)	4, 25, 27, 30, 40
CM cellulose	$-COO^-$	Cellulose	Transaminase (58); egg white (53)	24
Anion exchangers				
DEAE cellulose	$-N^+H(C_2H_5)_2$	Cellulose	Serum (59); rattlesnake venom (*12*)	23, 43, 58
TEAE cellulose	$-N^+(C_2H_5)_3$	Cellulose	Horse-radish enzymes (*13*)	48
Dowex 2	$-N^+(CH_3)_2 \cdot C_2H_4OH$	Polystyrene	Red-cell phosphatase, serum albumin (*10*)	*9*, 35, 54
Amberlite IRA-400	$-N^+(R)_3$	Polystyrene	Accelerator globulin (20)	

B. The first application of an ion exchanger to protein work was when Paleus and Neilands in 1950 used the carboxylic acid resin, IRC 50, for the purification of cytochrome c (41). One year later, Hirs, Moore, Stein and Tallan published the first of a series of careful studies on different proteins on the same resin (26, 27, 61, 62). By now, the number of ion exchangers used for protein chromatography is rather large, but a survey can be obtained, even here, from the table in the review of Sober and Peterson (60).

Ion exchangers are conventionally classified according to their functional groups and the nature of the skeleton. A detailed monograph on ion exchangers has been published by Samuelson (55), and more recent contributions to both theory and applications are found in the volume edited by Calmon and Kressman (17) and in the monograph of Kunin (31).

Some of the ion exchangers used for protein chromatography have been listed in Table I. As the table, however, does not show anything about the number of applications, it ought to be emphasized that Sober and Peterson's introduction of the cellulose derivatives as adsorbents (43, 58) was a particularly large stimulus to the development of chromatography of proteins.

[1] The following abbreviations are used in the present paper: CM for carboxymethyl, DEAE for diethylaminoethyl, DNA for deoxyribonucleic acid, DNAase for deoxyribonuclease, SE for sulfoethyl, SM for sulfomethyl, TEAE for triethylaminoethyl and THAM for tris(hydroxymethyl)aminomethan.

1. *Experimental details*

The equipment for chromatographic work, such as columns and fraction collectors, is described in the handbooks of Turba (68) and of Lederer and Lederer (34). Only a few points, which are of special interest in protein chromatography, will be mentioned in the following.

The column.—In starting-agent development, both the length and uniformity of the packing of the column are important for the resolution. In one-step development, especially in the presence of displacement effects, it is necessary to obtain sharp and straight fronts of the zones. Thus, in both these cases, some attention must be paid to the packing, and experience shows that it is convenient to have a ratio of about 1/20 (or lower) between the diameter and the length of the column. In stepwise and gradient development, when the length of the step or the gradient respectively is relatively large when compared to the fraction volumes, the dimensions and the uniformity of the column are of less importance for the resolution.

The developing agent.—In liquid-solid chromatography of proteins, the developing agents have so far always been buffer solutions. Especially when the buffer ions can interfere with the adsorbents, as in the case of ion exchangers, some less desirable phenomena can occur if the following points are not considered:

1. It is necessary to check that the adsorbent is in equilibrium with the last washing agent used (in the following referred to as the starting agent or the starting buffer). This may appear too trivial to be stressed, but experience has shown the necessity of paying attention to this point. In the case of the very low ionic strengths used with some adsorbents, equilibration takes a considerable time and checking must not be done with paper indicators—the buffer capacity of the paper itself is too high. It is possible to work with a non-equilibrated column (19) or without buffers (20), but no doubt the results are improved by working under as well defined conditions as possible.

2. With the exception of starting-agent elution, the best pH control is obtained by using cation buffers on anion exchangers and anion buffers on cation exchangers (*9*, *10*).

3. The introduction of new ions should be avoided if possible. As shown in gradient elution on a cation exchanger (*8*), the introduction of another anion may produce an out-coming gradient deviating from the theoretical one. A possible explanation may be adsorption of the ions also to the skeleton. The existence of such and similar phenomena has been described by Reichenberg and Wall (52).

II. Starting-agent development

This is defined as development entirely with the same agent as used for the equilibration of the column. There are several advantages to this procedure: In ideal cases separations are based on the differences between the partition coefficients only, and this permits a theoretical approach and the calculation of R_f values. The distribution can be either between two liquid phases, as in the cellosolve-salt-water system of Martin and Porter (37), or between a liquid and a solid phase, for instance an ion exchanger.

The starting-agent development is the most satisfactory way to investigate the chromatographic homogeneity of a protein. Rechromatography on the same column

would produce a peak in the chromatogram with the same R_f value as in the first experiment.

The resolution efficiency of the starting-agent development can be illustrated by the separation of the different pancreatic ribonucleases, independently carried out by Martin and Porter (37), and by Hirs, Moore and Stein (26). The two enzyme forms have later been shown to differ only by one carboxylic group (63). This type of ion exchange chromatography was later used by the Rockefeller Institute group, not only with ribonuclease (26), but also with lysozyme (61), chymotrypsinogen (27) and trypsinogen (62), and by Boardman and Partridge for the separation of hemoglobins from different species (4).

The most serious disadvantage of the starting-agent development is, however, that it works in very few cases only. It has also been pointed out by Boardman and Partridge (4) and by Jollès and Fromageot (30) that the method is very sensitive toward changes in pH and ionic strength, and in the case of partition chromatography, also toward temperature (50). The large dilution obtained is also a disadvantage, especially as surface denaturation must be considered to occur for many proteins (cf. (*15*)). A natural consequence of the high selectivity is that it is difficult to recover very different proteins in the same chromatographic experiment.

If a protein has a R_f value near 1, but the impurities are retarded, this can, according to the definition, be called starting-agent development. It appears that Dowex 2 can give satisfactory purifications in this way. Some experiments with peroxidase were published (*10*) and passage through a column of Dowex 2 was used as a step in a simplified purification of this enzyme, recently described by Paul (42). Huisman *et al.* (28) have reported the isolation of a heat-coagulable globulin from urine of a patient with multiple myeloma during similar conditions, although, in this case, step-wise development was applied in order to recover other fractions. Also pancreatic lipase can be purified in the same way (Borgström, personal communication) and the method may be especially useful for easily denaturated enzymes.

III. Gradient development

This is defined as development with an agent, the composition of which is changed continuously from the starting agent toward a solution of higher eluting power.

The technique was first discussed by Alm *et al.* (2), although it had been used earlier by Mitchell *et al.* (38). Different ways to produce desired gradients have been described by Desreux (21), Bock and Ling (6), Lakshmanan and Lieberman (32), and Drake (22).

The results from a gradient development will depend upon whether the outcoming gradient of the column is the same as the one leaving the mixing chamber. To achieve this, it is necessary to choose a developer which does not interact with the adsorbent. In the case of ion exchangers this means that one has to observe the following conditions: (*a*) to work outside the titration range of the adsorbent (*8*), (*b*) to avoid the introduction of new ions (*8*), and (*c*) to select the buffer according to an earlier discussion (*10*).

Whenever a substance in the developer interacts with the column material, an attempt to produce a certain gradient of this substance will lead to a sharpening of the gradient or to a step. Thus to neglect the conditions just mentioned may lead

to losing the idea of the *continuously* changing developer, and the result may be a decreased resolution or—still worse—artifacts, such as double zoning. If, for instance, a gradient of a new anion is introduced on DEAE cellulose (23, 59) this will be subject to frontal analysis and may at its break-through very likely give rise to an increased protein concentration in the same way as in step-wise development (cf. p. 10).

The types of gradient used in protein chromatography involve changes in pH (*8*), in ionic strength or combinations of both (23, 59). Methods to produce pH gradients have been subject to a theoretical investigation by Piez (46).

A simple device for a concave pH gradient has been used for a purification of prostatic acid phosphatase on the sulfonic acid resin, Dowex 50 (*8*), and the same system has later been used by Pierpoint for the separation of some vegetable phosphatases (44, 45).

As the adsorption properties of proteins are often drastically changed with small differences in the buffers (66), gradient elution would be expected to offer good separation possibilities for proteins (22). However, this assumes that only a small part of the capacity of the column is utilized and that the length of the gradient is large compared with the dead volume of the column. The first condition is sometimes difficult to obtain as the capacity for some adsorbents, especially the resins, often must be utilized to its upper limit (*10*). The latter fact can sometimes be slightly inconvenient, as in the serum fractionation by Sober *et al.* (59) where the elution was carried out during about 200 hours. Thus, for preparative purpose, the use of gradient development with a complex protein mixture may result in a pattern of overlapping zones, which may be difficult to interpret unless one or more among the components may be determined by a specific reaction, such as an enzyme activity.

If the outcoming gradient is analysed, a single experiment may, however, yield information which makes unnecessary a more laborious trial-and-error procedure for the finding of the optimal conditions for a step-wise or starting-agent development (*8*, 22).

It is possible to use gradient elution to obtain information about the homogeneity of certain proteins (*10*, 40). For this purpose, it is necessary to follow two properties of the protein, one as specific as possible (for instance, an enzymatic activity), the other as general as possible (for instance, ultraviolet extinction). In this way, gradient elution on Dowex 2 has been used to investigate the homogeneity of purified cysteinyl-glycinase (56). Gradient elution can also be used to resolve a spectrum of closely related molecules as shown by Tiselius *et al.* (67) in their subfractionation of human γ-globulin with calcium phosphate. This chromatogram is rather similar to the one obtained by Porter when using partition chromatography (50).

As a homogeneity criterion, gradient development does, however, not offer the same resolution as the starting-agent development. The analysis of the specific activity during a gradient development may, possibly, permit the detection of an inactive contamination in an enzyme preparation, but a micro-heterogeneity in the activity may be overlooked as shown in the study of lysozyme by Gonçalves *et al.* (25).

IV. Step-wise development

This is defined as development with an agent which is different from the one used for the equilibration of the column. In protein chromatography this is probably the method most often used. It is very simple to perform such an experiment, but the

mechanism is rather complex and more pitfalls are involved than in the other methods of development.

For practical reasons, one may distinguish between the case when the interaction between developer and adsorbent is negligible, and when there is an evident interaction between developer and adsorbent. The material has therefore been organized into the following three paragraphs:

1. *One step with negligible interaction between developer and adsorbent*

In studying the separation of horse-radish peroxidase and acid phosphatase on Dowex 2, it was observed that a slight overloading gives rise to small amounts of phosphatase on the back side of the peroxidase zone (for instance, in chromatograms like Fig. 9 in (*10*)). In order to investigate this phenomenon, the fraction volumes were reduced by a factor of ten down to about 1/100 of the dead volume of the column, and elution was directly carried out with a buffer concentration sufficient to desorb almost all the material in the enzyme mixture. It was found that this procedure separates peroxidase and acid phosphatase into adjacent narrow zones (see Fig. 10 (*10*)). Moreover, the phosphatase zone showed a minimum which indicated a heterogeneity, later shown to be due to the presence of two different enzymes.

According to Tiselius (64) ideal displacement is characterized by the fact that the length of the zone is proportional to the amount of material applied and the concentration is dependent only on the affinity constant of the substance and the concentration of the displacer.

The effect of the load was first studied on Dowex 2 (*10*) and then, in greater detail, on TEAE cellulose (*13*). It was found that when increasing amounts of horse-radish extracts were applied to the column, the peaks of the peroxidase and the first acid phosphatase became broader, while the peak of the second phosphatase increased only in height. The explanation was suggested that the separation mechanism for peroxidase and the first phosphatase was influenced by mutual protein displacement, in contrast to the case of the second phosphatase, which appeared to be eluted by the chloride ion gradient (*13*).

However, a major objection to these experiments as indications for the existence of mutual protein displacement lies in the fact that increasing amounts of material were dissolved in increasing volumes of buffer (*13*). It might also be possible to regard these one-step developments as very steep gradient experiments and interpret the separation as entirely due to the increasing chloride concentration.

It was, therefore, thought to be of interest to demonstrate a pure protein-protein displacement (14) in a somewhat similar manner as initially used by Polis and Shmukler (47). The experiments were planned to show that a moderately adsorbed enzyme, prostatic acid phosphatase, can be desorbed by another more strongly adsorbed protein even if the salt concentration was kept constant throughout the run. Dowex 2 was chosen as adsorbent, because of the fact that elution data were already known for a variety of proteins for this resin (*10*). One mg of freeze-dried prostatic extract was dissolved in 1 ml of THAM-HCl buffer of pH 7.3 and applied to a column with 19 ml of resin, and development was directly started with a 0.1 % solution of α_2-globulin (human serum) dissolved in the same buffer. Fig. 1 shows three such experiments, carried out in the same way but with different constant concentrations of the THAM–HCl buffer. The upper part of the figure shows the results with the

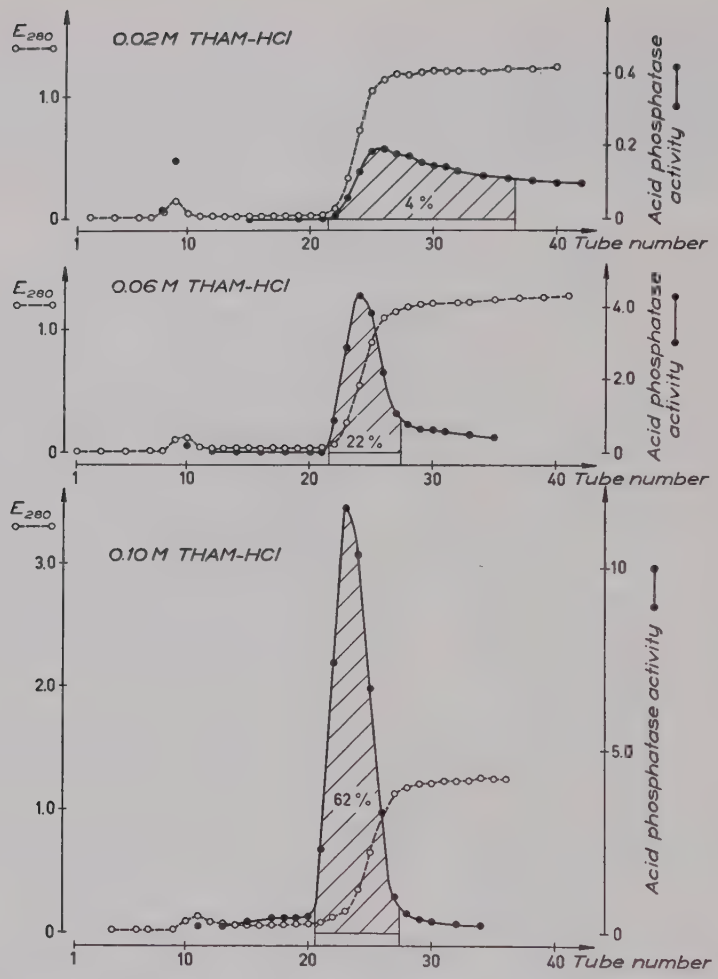

Fig. 1. Displacement of prostatic acid phosphatase by human serum α_2-globulin at three different constant salt concentrations. Column with 19 ml of Dowex 2. Fraction volumes about 1 ml.

lowest buffer concentration, 0.02 *M*. Only small amounts of phosphatase (recovery 4 %) were eluted as a very wide-spread zone, and the front coincides with the front of the developer. As a whole, the chromatogram looks like an unsuccessful frontal analysis. However, when the adsorption of the phosphatase is decreased by increasing the salt concentration to 0.06 *M*, 22 % of the phosphatase activity could be recovered as a relatively sharp zone, localized in the middle of the front of the developing α_2-globulin (Fig. 1, middle part). A further increase in the buffer concentration to 0.10 *M*, gave a recovery of 62 % phosphatase as a very sharp zone in the beginning of the front of the developer (Fig. 1, lower part). In all the three chromatograms the small peak around tube 10 is due to traces of unadsorbed material.

These experiments demonstrate that mutual protein displacement exists but that too strong adsorption will prevent a separation based on displacement.

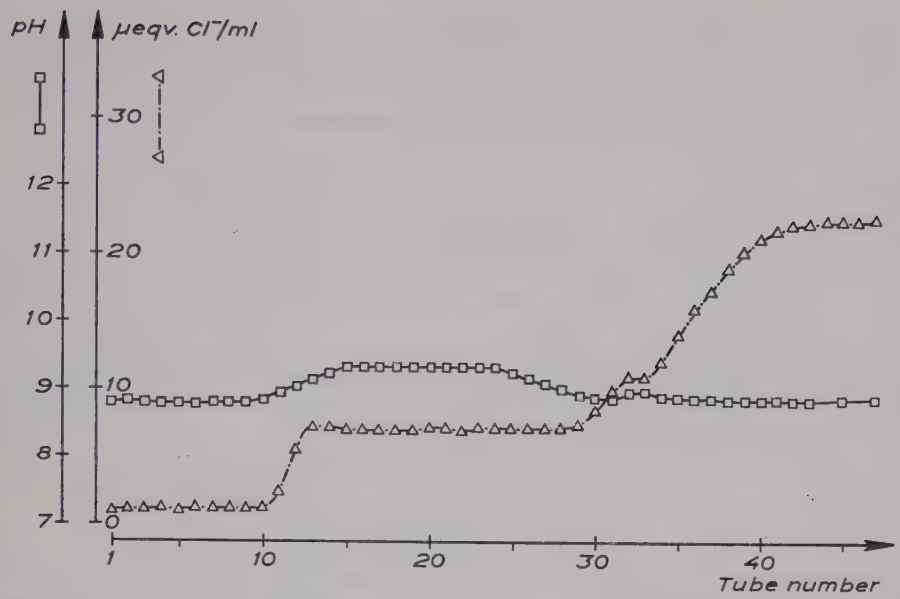

Fig. 2. Chloride and pH variations in the effluent from a column of 19 ml of DEAE cellulose without any substance. The column was equilibrated with 0.005 *M* THAM–HCl buffer of pH 8.9, and from the beginning of the experiment (at tube number 1) the column is washed with 0.10 *M* THAM–HCl buffer of pH 8.9. Fraction volumes around 1 ml and flow rate about 17 ml per hour.

2. *One step with evident interaction between developer and adsorbent*

In the case of ion exchangers, the adsorption of the developer occurs when working near the pK of the adsorbent, or when new ions are introduced. As starting-agent development on the carboxylic acid resins has often proved inconvenient or impossible, a one-step development has been used in several cases, as discussed by Moore and Stein in their review (39).

The behaviour of some enzymes in rattle snake venom has been studied in this way (*11*, *12*) using the DEAE cellulose of Peterson and Sober (43). The load on the column was kept constant while the pH and ionic strength of the developer were varied. In this case the resolution was gradually improved by bringing pH near the pK of the cellulose ion exchanger and by decreasing the concentration of the developer. Under optimal conditions, two or three different modifications of phosphodiesterase could be separated from lecithinase and amino acid oxidase. With DEAE cellulose, no displacement phenomena could be observed when the load was varied.

In the case of the step-wise development of serum albumin on the quaternary ammonium base, Dowex 2, (*10*), it was found that each shift of elution buffer would produce a corresponding step in the chloride curve for the effluent (see Fig. 3). Also on TEAE cellulose, a single step would give rise to a corresponding single step in the chloride curve (see Fig. 5 in (*13*)). For the tertiary ammonium base, DEAE cellulose, it has, however, recently been found that *one* shift in the elution concentration can give rise to *two* corresponding steps in the chloride curve as shown in Fig. 2 (3). The explanation is possibly connected with the complicated set of equilibria which must exist for the tertiary ammonium bases. In any case it is obvious that

this phenomenon involves risks for misinterpretation of the chromatograms from step-wise developments. It is possible that the rattle snake venom may contain only two different phosphodiesterases instead of three as suggested earlier (*12*), the third one being an artifact produced by the phenomenon just mentioned.

3. *Development with several steps*

From the separation of low-molecular-weight substances with curved isotherms, it is well known that development with several steps may give rise to several zones with the same substance, and, in fact, this was one of the arguments used for the introduction of gradient development (2).

The chromatographic behaviour of proteins appears, however, due to their narrower elution ranges, to be quite different from that of low-molecular-weight substances (66). Step-wise development had also proved very promising, first on calcium phosphate (66) and later on Dowex 2 (*9*), and it was therefore found necessary to make a more detailed study of the procedure (*10*).

The adsorbent used was Dowex 2 and the protein was human serum albumin, but very likely the results would apply to the behaviour of other proteins also on other adsorbents. The developers used were THAM–HCl buffers and as pH was constant (7.3), the chloride and the protein were the only anions which could vary. In order to facilitate the interpretations, all these variables were followed in the effluent. It was found that the buffer concentration at which a protein is completely desorbed is a constant, but that the first concentration at which it can be partly eluted depends on the amount applied to the column. If intermediary steps are introduced, each step of chloride ions will give rise to a zone of serum albumin as shown in Fig. 3. The explanation was suggested that the capacity of the resin for protein adsorption decreases continuously with the increasing ionic strength (*10*). As a consequence, a protein zone is spread out over the column with increasing buffer concentration, and when the whole column is covered, the protein begins to appear in the effluent.

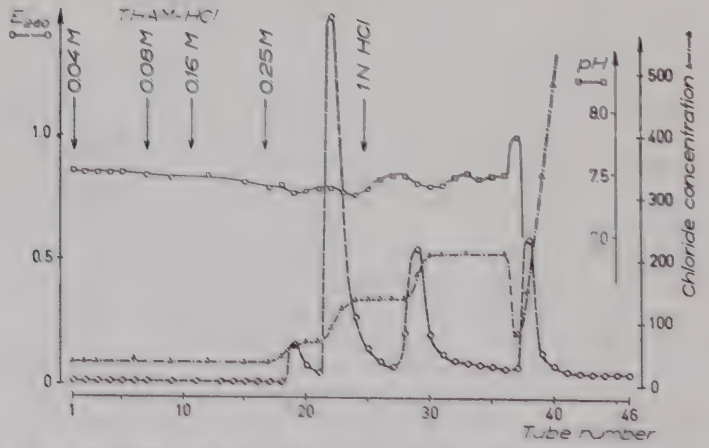

Fig. 3. Step-wise development of 60 mg of human serum albumin with a column (48 × 2.2 cm) of 180 ml of Dowex 2. Fraction volumes about 6.2 ml. The arrows indicate the start of elution at the top of the column with the different agents.

It was also pointed out that this behaviour of the protein was in good agreement with the results expected when a substance with a curved adsorption isotherm is desorbed by a weak eluting agent (*10*).

It is clear that for the separation of a mixture of two or more proteins, the use of step-wise development may result in a chromatogram with the same substance in several zones as well as several substances in the same zone. On the other hand, if the load and the elution conditions are chosen with care, very good separations can be obtained (see Fig. 4 and ref. *10*, *15*, 53, 56). The procedure may also have some analytical applications, as in the separation of different pathological hemoglobins (29), the subfractionation of serum albumin (67) and the analysis of albumin denaturated with gamma rays (54).

The separation mechanism may in some cases be due to the fact that the elution concentrations for the different substances are different enough to permit an almost complete separation, as in the cases of hemoglobin and red-cell phosphatase (*10*), but very likely displacement phenomena facilitate many separations carried out by step-wise elution.

V. Chromatographic homogeneity

A protein is said to show chromatographic homogeneity if no further resolution can be obtained during optimal separation conditions, and to show heterogeneity if it can be shown to contain fractions with different chromatographic properties. The practical difficulty in the demonstration of homogeneity is to find the optimal separation conditions; in the case of a heterogeneity the difficulty is to avoid artificial double zoning, caused for instance by some of the factors discussed below.

As mentioned earlier, the starting-agent development is the most satisfactory way to investigate the chromatographic homogeneity of a protein (p. 4), but also gradient development can give information about the purity of an active protein (p. 6). The step-wise development will involve too sudden changes in the adsorption behaviour to permit conclusions about homogeneity.

The significant difference between development with one single step and several steps lies in the fact that the first procedure should give rise to only one single increase in the concentration of the eluting substance. The advantage would be that, if the column were not overloaded, and if the concentration of the displacer were high enough, a single substance should not be able to give rise to more than one zone in the effluent. Thus, if two zones of the same enzyme activity appear in a chromatogram of a one-step development (*12*, *13*), it may be concluded that this is due to the existence of two different forms of the enzyme. In order to exclude artifacts as far as possible, it is, however, always necessary to rechromatograph the different fractions in the same way as in the first experiment.

1. *Artifacts*

The risk of obtaining double zoning (the same substance in two or more zones) in column operations has been subject to surprisingly little discussion, in view of the very extensive literature on chromatography. Some special conditions which may cause double zoning in partition chromatography have been reviewed by E. Lester Smith (57), but among the 3704 references given by Lederer and Lederer (34), only four other papers seem to deal with double zoning.

As the question of micro-heterogeneity of enzymes appears to attract an increasing attention, it may be justified to list a number of factors which can produce double zoning in ion exchange chromatography of proteins:

1. The load on the column. As most workers are interested in using chromatography for preparative purposes, the risk for overloading may be apparent. Except in the case of the starting-agent development where a low load is a necessity, overloading will give rise to a zone with very little or no retention by the column. On the other hand, it has been found that too low a load may not give the optimal resolution of a complex enzyme mixture (*13*). Too high a protein concentration may, after the separation on the column, give rise to zones with different pH and in that way produce artificial fractionations.

2. Too low a concentration of the developer. In step-wise development this may produce chromatograms like the one in Fig. 3 and in all types of development, the risk exists that a relevant fraction is left on the column.

3. Denaturation of the proteins during the run. This has been reported by, among others, Boardman and Partridge for hemoglobin (4) and Ellis and Simpson for growth hormone (23).

4. Complex formation. This may occur between different and similar proteins, between proteins and low-molecular-weight organic compounds, and between proteins and salts. Especially the last combination may be difficult to detect in rechromatography if, for instance, extensive dialysis must for some reason be avoided. Other consequences of complex formation are discussed by Moore and Stein (39).

5. Neglecting the "buffer rules", discussed elsewhere (p. 4). This may sometimes be found unavoidable and still efficient purification may be obtained, but even if the effluent is analysed for the relevant ions, an interpretation of the mechanism can be expected to be very complicated.

6. Other phenomena related to changed or disturbed equilibria between developer and adsorbent. Such a phenomenon was described in the discussion of the step-wise development (p. 9) and may be more frequent due to the low buffer concentrations at which many protein chromatographic experiments must start.

VI. Concluding remarks

Most workers in the field of protein chromatography have been interested in applying the method to a given problem, and thus they have mostly been satisfied as soon as they have found one adsorbent, which works reasonably well. Those who have been interested in the mechanism have chosen to study different proteins on the same adsorbent. Although consecutive chromatographic runs on different adsorbents have been used (*15*, 24, 35, 56), the behaviour of the material has rarely been studied during comparable conditions. Despite these facts, a few comments may be made.

As soon as the work has a preparative aspect, capacity will be a major problem in protein chromatography. The introduction of the cellulose derivatives by Sober and Peterson was a considerable improvement, but the low ionic strength at which the runs must start causes difficulties for a number of globulin materials sometimes precipitated during the preceding dialysis. The resins can be operated at higher salt concentrations but the advantage offered by this condition is counterbalanced by their much lower capacity (*10*). An interesting attempt to improve the capacity of

a resin has been made by Boardman (5), who prepared a sulfonated polystyrene on kiselguhr. The capacity of this adsorbent was about 10 mg of insulin/ml wet resin (Boardman, personal communication).

The successful application of chromatography to the purification of an enzyme is dependent not on the behaviour of the enzyme only, but also to a large degree on the properties of the impurities. The ideal solution would thus be to prepare an adsorbent specific only for the enzyme. An interesting attempt in this direction was made by Malmström (36), who has purified a magnesium-activated enzyme, enolase, by adsorption on the magnesium form of SM cellulose (48). A further extension would be to study the behaviour of enzymes on columns of ion exchangers prepared in the substrate form.

CHROMATOGRAPHIC STUDIES OF SOME PHOSPHOESTERASES

Protein chromatography can be applied in enzyme research for at least three different purposes: as a purification step, as a homogeneity criterion, and as a method to obtain information about the substrate specificity. The first two of these aspects have been discussed earlier (*10*) and in the first part of this paper. The use of chromatography for studying the substrate specificity of an enzyme is in principle similar to the study of the homogeneity. If a separation process distributes the material of an enzyme preparation into a number of fractions, and if the activities toward two substrates are distributed over these fractions in the same way, this indicates that the same enzyme is responsible for the action. In the following a summary will be made of this and other applications of chromatography to the study of some phosphomono- and phosphodiesterases.

I. Acid phosphomonoesterases

Numerous attempts have been made to purify an acid phosphatase and a variety of different starting materials have been investigated. The author has worked with the enzymes obtained from white mustard seed (Anagnostopoulos and Boman, unpublished work), from horse-radish (*10*, *13*), from red cells (*10*) and from human semen (7, *8*) and prostate (*8*, *15*).

During the work with the horse-radish phosphatase, it was found that the activity was heterogeneous (*10*) and the preparation was later resolved into two enzymes with different chromatographic but similar enzymic properties (*13*). A preparation of another vegetable acid phosphatase was simultaneously investigated by Pierpoint (44, 45). He obtained a considerable purification of two similar enzymes by gradient elution on Dowex 50, and the same technique indicated that both the phosphatase and the metaphosphatase activities were due to the same enzyme.

A comparison of different starting materials for the preparation of an acid phosphatase showed that human prostatic tissue and semen contained 100–1000 times more activity than any of the other materials tried. Assuming the turnover numbers to be of similar magnitude, a purification starting from prostatic tissue could thus be kept within reasonable limits and still possibly yield measurable amounts of a pure enzyme. Special interest may also be devoted to the prostatic phosphatase, due to the fact that this enzyme is characteristic of humans and higher apes, it is

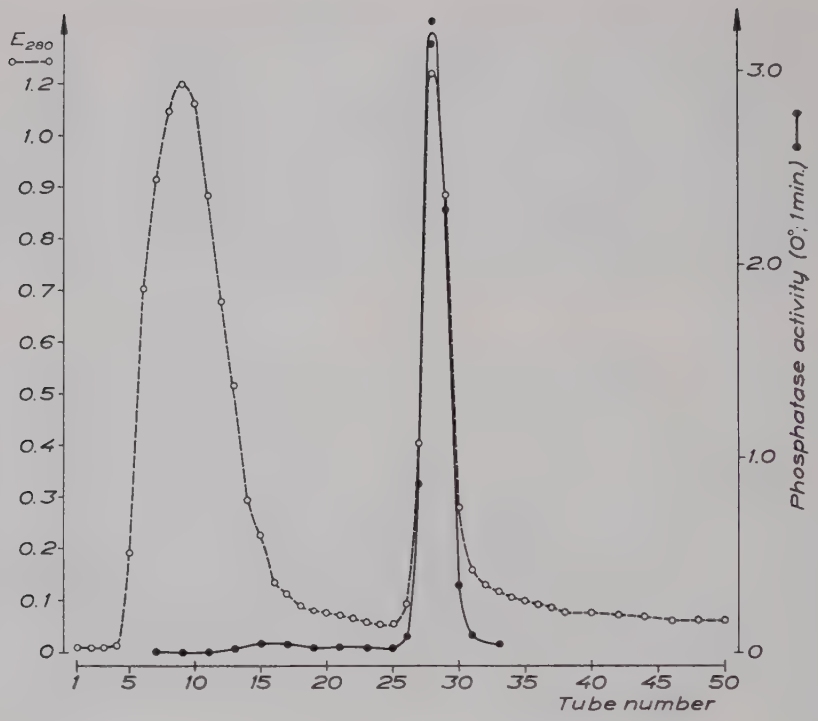

Fig. 4. Step-wise development of 600 mg of prostatic extract with a column (72 × 3.3 cm) of 600 ml of Dowex 50; X-2. Fraction volumes about 6.2 ml. The second zone (tube 28) contains purified acid phosphatase, homogeneous in ultracentrifuge (see Fig. 5) and column electrophoresis.

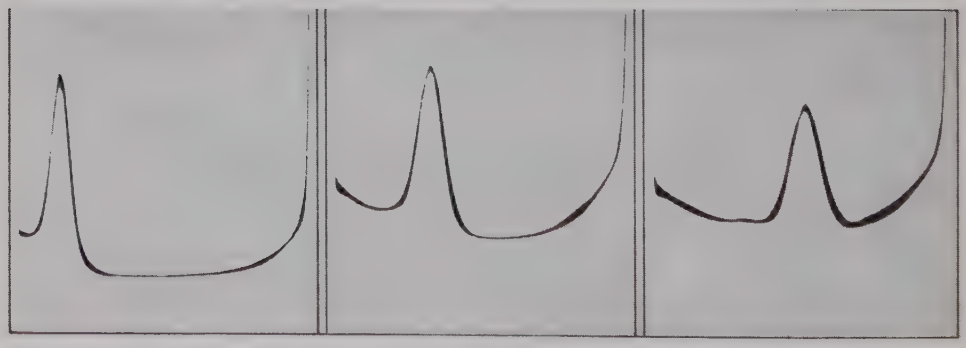

Fig. 5. Ultracentrifugal pattern of prostatic acid phosphatase, purified by chromatography on Dowex 50 (tube 28 in Fig. 4). The first exposure was taken 26 min. after full speed (59,780 r.p.m.), the second one after 58 min. and the third one after 90 min. The extinction at 280 mμ of the solution was 9.43, and the sedimentation constant obtained was 5.6 *S*.

connected with prostatic cancer and it is frequently used as a reagent for terminal phosphate groups. Moreover, chromatography on Dowex 50 had proved to be a very efficient purification step for prostatic phosphatase (*8*). These factors were considered to balance the disadvantages of a pathologically heterogeneous starting material.

By a systematic variation of the extraction conditions and the chromatographic technique, a two-step preparation was found which gave a pure phosphatase (*15*). Fig. 4 shows a preparative chromatogram from a column of Dowex 50 with 600 mg of prostatic extract. The second zone (tube 28) contains the acid phosphatase, which was found to be homogeneous when investigated by ultracentrifugation (see Fig. 5) and column electrophoresis.

II. Phosphodiesterases

Phosphodiesterases have been far less studied than monoesterases. However, during the last few years an increasing number of papers have been dealing with diesterases digesting nucleic acids. The work of the author has been limited to the study of two different enzymes, both of them hydrolyzing deoxyribonucleic acid (DNA).

One of these enzymes is present in snake venoms and was considered to act on oligonucleotides, but not on macromolecular DNA (24). The enzymic action on DNA was then followed by the viscometric method. However, estimation of the deoxyribonuclease (DNAase) activity, as the increase in the lanthanum-acid soluble material, showed the presence of a considerable alkaline DNAase activity. A chromatographic fractionation of rattle snake venom on DEAE cellulose demonstrated the existence of at least two different diesterases, both of them with DNAase activity (*11*). A more detailed investigation showed the chromatographic fractionation to remove amino acid oxidase and lecithinase and the heterogeneity of the diesterase activity was established by rechromatography (*12*). The apparent contradiction with respect to the activity toward macromolecular DNA was resolved by assuming the snake-venom diesterase to be an exonuclease (33). This hypothesis has very recently been confirmed by Heppel and by Khorana during experiments with enzymatically synthetised polynucleotides of known structure (Laskowski, private communication).

The second diesterase studied was found in human prostatic tissue in connection with the work on the purification of the acid phosphatase. It was separated from the acid phosphatase by column electrophoresis and a further purification was obtained by chromatography on DEAE cellulose (*16*). The enzyme has pH optimum around 5.3 and causes a depolymerisation of the DNA. It resembles in these respects other "intracellular" DNAases. However, other DNAases seem to require a divalent cation for full activity, but for prostatic DNAase no evidence was found for a metal-ion activation. Thus this enzyme may prove to be a useful complementary tool for structure investigations on DNA. The present purification should, however, be regarded as only a preliminary attempt, and it is hoped that a more detailed study of this enzyme may be pursued.

SUMMARY

The first part of the paper is an analysis of the different methods of development used in ion exchange chromatography of proteins. A discussion is also given of several risks for misinterpretations of chromatograms. The second part is a brief review of applications to the study of some phosphoesterases.

Acknowledgements

I wish to express my sincere gratitude to Prof. A. Tiselius for his always stimulating interest and for much helpful advice throughout the progress of my work. I am indebted to many of my colleagues at this Institute and especially to Dr. B. G. Malmström for innumerable discussions on different aspects of enzyme chemistry and for valuable criticism on almost all my manuscripts. Most of my work here summarized has been supported by the Swedish Cancer Society and the Rockefeller and the Wallenberg Foundations.

REFERENCES

1. Agner, K., Biochem. J. *32*, 1702 (1938).
2. Alm, R. S., Williams, R. J. P. and Tiselius, A., Acta Chem. Scand. *6*, 826 (1952).
3. Björk, W. and Boman, H. G. (to be published).
4. Boardman, N. K. and Partridge, S. M., Biochem. J. *59*, 543 (1955).
5. Boardman, N. K., Biochim. et Biophys. Acta *18*, 290 (1955).
6. Bock, R. M. and Ling, N.-S., Anal. Chem. *26*, 1543 (1954).
7. Boman, H. G., Nature *173*, 447 (1954).
8. Boman, H. G., Biochim. et Biophys. Acta *16*, 245 (1955).
9. Boman, H. G., Nature *175*, 898 (1955).
10. Boman, H. G. and Westlund, L. E., Arch. Biochem. Biophys. *64*, 217 (1956).
11. Boman, H. G. and Kaletta, U., Nature *178*, 1394 (1956).
12. Boman, H. G. and Kaletta, U., Biochim. et Biophys. Acta *24*, 619 (1957).
13. Boman, H. G. and Westlund, L. E., Arch. Biochem. Biophys. *70*, 572 (1957).
14. Boman, H. G., in "Symposium on Protein Structure" (Ed.: Neuberger, A.), p. 100, Methuen & Co., London 1958.
15. Boman, H. G., Arkiv Kemi *12*, 453 (1958).
16. Boman, H. G., Arkiv Kemi *12*, 467 (1958).
17. "Ion Exchangers in Organic and Biochemistry" (Eds.: Calmon, C., and Kressman, T. R. E.), Interscience Publ. Inc., New York 1957.
18. Cassidy, H. G., "Fundamentals of Chromatography", Interscience Publ. Inc., New York 1957.
19. Cochran, G. W., Chidester, J. L. and Stocks, D. L., Nature *180*, 1281 (1957).
20. Cox, F. M., Lanchantin, G. F., and Ware, A. G., J. Clin. Invest. *35*, 106 (1956).
21. Desreux, V., Rec. trav. chim. *68*, 789 (1949).
22. Drake, B., Arkiv Kemi *8*, 1 (1955).
23. Ellis, S. and Simpson, M. E., J. Biol. Chem. *220*, 939 (1956).
24. de Garilhe, M. P. and Laskowski, M., Biochim. et Biophys. Acta *18*, 370 (1955).
25. Gonçalves, J. M., Molinari, R., and Deutsch, H. F., Arch. Biochem. Biophys. *60*, 171 (1956).
26. Hirs, C. H. W., Moore, S. and Stein, W. H., J. Biol. Chem. *200*, 493 (1953).
27. Hirs, C. H. W., J. Biol. Chem. *205*, 93 (1953).
28. Huisman, T. H. J., van der Wal, B., Groen, A. and van der Sar, A., Clin. Chim. Acta *1*, 525 (1956).
29. Huisman, T. H. J. and Prins, H. K., Clin. Chim. Acta *2*, 307 (1957).
30. Jollès, P. and Fromageot, C., Biochim. et Biophys. Acta *19*, 91 (1956).
31. Kunin, R., "Ion Exchange Resins", John Wiley & Sons Inc., New York 1958.
32. Lakshmanan, T. K. and Lieberman, S., Arch. Biochem. Biophys. *53*, 258 (1954).
33. Laskowski, M., Hagerty, G. and Laurila, U.-R.; Boman, H. G., Nature *180*, 1181 (1957).

34. Lederer, E. and Lederer, M., "Chromatography. A Review of Principles and Applications", Elsevier Publ. Co., Amsterdam 1957.
35. Malmström, B. G., Levin, Ö. and Boman, H. G., Acta Chem. Scand. *10*, 1077 (1956).
36. Malmström, B. G., Arch. Biochem. Biophys. *70*, 58 (1957).
37. Martin, A. J. P. and Porter, R. R., Biochem. J. *49*, 215 (1951).
38. Mitchell, H. K., Gordon, M. and Haskins, F. A., J. Biol. Chem. *180*, 1071 (1949).
39. Moore, S. and Stein, W. H., Adv. Prot. Chem. *11*, 191 (1956).
40. Morrison, M. and Cook, J. L., Science *122*, 920 (1955).
41. Paleus, S. and Neilands, J. B., Acta Chem. Scand. *4*, 1024 (1950).
42. Paul, K. G., Acta Chem. Scand. (in the press) (1958).
43. Peterson, E. A. and Sober, H. A., J. Am. Chem. Soc. *78*, 751 (1956).
44. Pierpoint, W. S., Biochem. J. *65*, 67 (1957).
45. Pierpoint, W. S., Biochem. J. *67*, 466 (1957).
46. Piez, K. A., Anal. Chem. *28*, 1451 (1956).
47. Polis, B. D. and Shmukler, H. W., J. Biol. Chem. *201*, 475 (1953).
48. Porath, J., Arkiv Kemi *11*, 97 (1957).
49. Porter, R. R., Biochem. J. *53*, 320 (1953).
50. Porter, R. R., Biochem. J. *59*, 405 (1955).
51. Porter, R. R., in "Methods in Enzymology" (Eds.: Colowick, S. P. and Kaplan, N. O.) Vol. I, p. 98, Academic Press, New York 1955.
52. Reichenberg, D. and Wall, W. F., J. Chem. Soc. *1956*, p. 3364.
53. Rhodes, M. B., Azari, P. R. and Feeney, R. E., J. Biol. Chem. *230*, 399 (1958).
54. Rosen, D. and Boman, H. G., Arch. Biochem. Biophys. *70*, 277 (1957).
55. Samuelson, O., "Ion Exchangers in Analytical Chemistry", Almqvist och Wiksell, Stockholm 1952.
56. Semenza, G., Biochim. et Biophys. Acta *24*, 401 (1957).
57. Smith, E. Lester, Nature *169*, 60 (1952).
58. Sober, H. A. and Peterson, E. A., J. Am. Chem. Soc. *76*, 1711 (1954).
59. Sober, H. A., Gutter, F. J., Wyckoff, M. M., and Peterson, E. A., J. Am. Chem. Soc. *78*, 756 (1956).
60. Sober, H. A. and Peterson, E. A. in "Ion Exchangers in Organic and Biochemistry" (Eds.: Calmon, C. and Kressman, T. R. E.), p. 318, Interscience Publ. Inc., New York 1957.
61. Tallan, H. H. and Stein, W. H., J. Biol. Chem., *200*, 507 (1953).
62. Tallan, H. H., Biochim. et Biophys. Acta *27*, 407 (1958).
63. Tanford, C. and Hauenstein, J. D., Biochim. et Biophys. Acta *19*, 535 (1956).
64. Tiselius, A., Arkiv Kemi Miner. och Geol. *16A*, No. 18, 1 (1943).
65. Tiselius, A., Disc. Faraday Soc. No. 7, 7 (1949).
66. Tiselius, A., Arkiv Kemi *7*, 443 (1954).
67. Tiselius, A., Hjertén, S. and Levin, Ö., Arch. Biochem. Biophys. *65*, 132 (1956).
68. Turba, F., "Chromatographische Methoden in der Protein-Chemie", Springer-Verlag, Berlin 1954.

Printed April 1958

Uppsala 1958. Almqvist & Wiksells Boktr. AB 586122